New and Notable

An Elegant Way to Quantitatively Analyze Oligomer Formation in Solution

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The analysis of the oligomerization of proteins is an old challenge that has never lost its importance. To the contrary, a growing number of proteins have been found to oligomerize under the influence of ligand binding as a key to biological control. Likewise, an increasing number of diseases are related to aberrant protein oligo- and polymerization. In a previous issue of the *Biophysical Journal*, an elegant method is presented to quantitatively characterize this phenomenon, based on the application of fluorescence correlation spectroscopy (FCS) (1). FCS measures fluctuations in concentrations of fluorescent molecules due to Brownian motion; as such, it needs small observation volumes and low concentrations of fluorescently labeled molecules (i.e., nanomolar as achieved in the observation volumes of contemporary confocal microscopes (2)).

It is therefore useful to study protein associations at low concentration and allows for the determination of very high association constants. However, many proteins associate/dissociate in the micromolar or higher concentration regime. In FCS, this challenge can be overcome by using trace amounts of the fluorescently labeled protein in the presence of an excess of unlabeled protein, such that total protein concentrations much higher than nanomolar can be measured. An additional outcome of this scheme is that every oligomer contains only one label, so that the brightness remains the same for all oligomers, considerably simplifying analysis. This method has been applied to the oligomerization of tubulin (3), α-synuclein (4), tumor suppressor P53 (5), and many other proteins.

Alternatively, brightness analysis—photon-counting histograms as well as other forms—has also been used extensively to study a number of protein systems (6–9). Typically, in these approaches all proteins are labeled and the brightness of each oligomeric species should be proportional to its size. These methods are powerful but do not always make use of the complementary diffusion information and still rely on nanomolar concentrations of fluorescent particles.

In the article by Chakraborty et al., in a previous issue, a combination of the two techniques is presented by mixing labeled proteins with unlabeled ones at concentrations much higher than trace amounts. The method is used to elucidate the details of oligomerization of the AAA+ ATPase Rubisco activase (Rca), a protein whose self-association is important to its function. At low protein concentrations (between 50 and 300 nM), samples consisted of only labeled protein. FCS showed simple curves, with a diffusion coefficient (D_1) independent of concentration and close to what was expected for monomer Rca. Photon-counting histograms also showed no changes in brightness, confirming that Rca is monomeric over this range of concentrations.

However, at higher total protein concentrations (>550 nM), the FCS curves shift to longer timescales with increasing protein concentration, indicative of the oligomerization of the protein. Despite the presence of multiple oligomeric species at these concentrations, the FCS curves could be fit adequately with an equation for a single diffusing species, yielding an apparent diffusion coefficient (D_{app}), which represents a brightness-weighted-average diffusion coefficient of all species present. D_{app} was found to decrease in a continuous manner with increasing concentration, as has been observed previously for tubulin (3), α-synuclein (4), and p53 (5). Because of this, the relative concentrations of each of the oligomeric species could not be determined from simply fitting the autocorrelation curves. Thus, the authors developed a method of analysis using D_{app}/D_1 to determine the average size of the oligomers at each concentration of protein. The relationship between oligomeric size and diffusion coefficient was modeled in two ways:

1. Assuming spherical symmetry, which leads to $D_k/D_1 = k^{-1/3}$ where k is the number of monomers in the oligomer, or
2. based on the calculation of the radius of gyration using x-ray crystallographic data.

It is worth noting that the first case is only approximately correct because a spherical dimer cannot have a radius that is 2× that of a monomer, and that for irregular shapes, D can also be calculated using the Hydrosub program (10). (The effect of shape on D is extensively discussed in the Supporting Material of the article by Chakraborty et al. (1).)

For further analysis, the authors derived an autocorrelation function to describe their polydisperse sample. This required assuming a well-defined diffusion coefficient and brightness for each oligomeric species, thus taking into account the fraction of labeled protein and the random distribution of labeled protein among the various oligomers. By estimating the D_k for each oligomer from x-ray crystallography data, the authors develop a fitting function where the only unknown parameter is the concentration of each oligomer, C_k, dependent upon the total protein concentration and the dissociation constants describing the equilibrium behavior of the oligomers. D_{app}/D_1 was plotted as a function of

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total protein concentration and this data was fit to their model. While a number of different scenarios are discussed, the best fit that could be obtained assumed the formation of dimers, tetramers, and hexamers with dissociation constants with 4, 1, and 1 μM, respectively, with the association into even larger species at very high protein concentrations (>10 μM).

Although hexamers have been identified previously by electron microscopy and mass spectrometry, and SE-HPLC elution profiles indicate a mix of oligomeric species, the measurements described here allowed for an analysis of the stoichiometry of the various oligomers that was previously unobtainable. This method should allow the quantitative study of allosteric regulation of Rca, which is of importance in the context of biological carbon fixation.

This method is very general and the equations are transparent and should have wide application to in vitro measurements. Its usefulness for measurements in the cell is, however, limited because concentrations cannot be varied at will, and the relation between size and diffusion coefficient is more complex in this crowded environment. Moreover, there are the known difficulties with the definition of the confocal volume in the inhomogeneous cellular environment (11). However, image correlation spectroscopy using brightness information seems to be able to give information on oligomer distributions (12,13) and heterospecies partition analysis for mixtures of different proteins (14).

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